SYNOPSIS

TITLE OF THE RESEARCH TOPIC

Development of pharmacologically active small molecular weight bioactives and polysaccharides from cephalopod mollusks belonging to the order Sepiida and Octopoda

NAME OF THE CANDIDATE

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CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
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KERALA

Synopsis

Title of the proposed topic	**:**	**Development of small molecular weight bioactives and polysaccharides from cephalopod mollusks belonging to the order Sepiidae and Octopoda**
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Background and Importance:

Marine derived bioactive components and the functional food ingredients with potential health benefits demonstrated to possess advantages as functional food with added health benefits. Novel secondary bioactive metabolites from the marine organisms are attracting attention because of the growing demand for new compounds of 'marine natural' origin, having potential applications in pharmaceutical fields, and concerns about the toxic effects by synthetic drugs and their derivatives.

The marine invertebrates, particularly molluscs were emerged as a prominent source of numerous unique secondary metabolites and could be used for the development of therapeutic drugs (Blunt et al., 2013; Faulkner 1999). Mollusks are one of the leading marine phylum, comprising around 23% of the named marine species (FAO, 2016), and cephalopods were found to constitute a major share among the marine-originated molluskan species. Cephalopods are considered as one among most healthy seafood, as less effected by environmental pollutants and culinary delicious and found to be a major share in the international market. The cephalopod molluscs were accounted for to be a rich source of protein content (Lee, 1994), as it stands today, 2% of humans' global consumption of protein is from cephalopods. They contain relatively low fat, high polyunsaturated fatty acids (PUFAs), essential amino acids, antioxidants and minerals, such as, selenium, which could not be acquired from other sources (Zlatanos et al., 2006). Cephalopod mollusks are traditional sea-foods with wide variety of bioactive secondary metabolites, and were considered to be valuable marine resources of nutritional and commercial importance (Chakraborty et al., 2020 ; Blunt et al., 2015). Among various molluscs, cephalopods are widely used in different parts of the world for various studies, but only recently they have been recognized as potential sources for bioactive compounds. The cephalopods represent important economic seafood for human consumption due to their nutritional and market value, cephalopod aquaculture has also shown an increase during the past few years. Among the marine molluscan species, the class *Cephalopoda* forms a major item in marine fishery export from India, with increasing demand from various parts of the world. They are the second major seafood export item from India to U.K, U.S.A and Burma. Narasimham et al., (1993) has described the cuttlefish resources, *Sepia aculeata* and *Sepia pharaonis* are the major species. *Sepia aculeata*, commonly known as the needle cuttlefish, is found in the fishery of all maritime states except Gujarat. The contribution of cephalopod to total Indian marine landing in 1993 was 4.3%. Kerala, Maharashtra and Gujarat together contribute 79% of total cephalopod landings of India (Menon et al., 1996). The soft-tissued cephalopods have been adapted to the adverse environments, and were believed that the cells of these sessile organisms are prolific producers of diverse classes of bioactive compounds and functional foods with biomedical significance (Blunt et al., 2017). Although these groups of compounds are homologous to those reported from terrestrial habitats, they are distinct with respect to their unusual chemical structures of rare occurrences and greater bioactivities, which could be attributed to their marine habitats. With innumerable edible cephalopod mollusks from the coastal ecosystem representing a large community of marine fauna, the south west coast of India are acknowledged for their particular richness. Considering the promising perspective for the utilization of these groups of organisms, and limited research reports on their utilization as potential health food, their pharmaceutical potential began to receive considerable attention. Increasing shares of patent application are from cephalopod-derived bioactive compounds and recent thrust of increased funding to develop marine nutraceuticals/pharmacophore

candidates appropriately signifies the importance of research in the area of bioactive molecules (Carroll et al., 2019). Considering the promising perspective for the utilization of these groups of mollusks, and limited research reports on their applications as potential health food, their pharmaceutical potential began to receive considerable attention. Although the percentage of marine natural products from cephalopod mollusks that could be developed into the drugs is low, it is clear that an increasing number of marine bioactive compounds will be approved for the treatment of human diseases.

Among the cephalopods, *Cistopus indicus, Amphioctopus marginatus, Amphioctopus aegina* belonging to the order *Octopoda*, and *Sepia pharaonis, Sepia elliptica,* and *Sepia aculeata* belonging to the order *Sepiidea* constitute an important part of marine fishery and aquaculture at the south west coast of India and are the preferred food items among the people living in this area. *I*n view of this, *these candidate species will be taken into account to isolate and characterize* pharmacologically active small molecular weight bioactives and polysaccharides, as well as to develop a library of molecules with bioactive potential. Optimized physical/chromatographic procedures will be developed to isolate and purify the target molecules of the lead bioactives. The in vitro bioactive properties of the isolated compounds will be assessed by their anti-oxidant, anti-inflammatory, antihypertensive, anti-inflammatory, anti-diabetic and anti-hypercholesterolmic *potential*. The isolated compounds will be characterized by exhaustive spectroscopic techniques, such as MS/IR/2D-NMR experiments, and novel leads would be validated through bioassay and structure-activity relationship analyses. This work also envisages the development of nutraceutical(s) and up-scaling their production towards their commercial utilization.

Objectives of the proposed work:

1) Development of process to isolate small molecular lead molecules and polysaccharides from cephalopods of marine origin by chromatographic techniques, coupled with evaluation of anti-hypertensive, anti-inflammatory, anti-dyslipidemic and anti-diabetic activities, using selective pharmacological models.
2) To characterize small molecular weight lead molecules and polysaccharide compounds from the extracts/fractions by extensive spectroscopic experiments, such as infrared (IR), mass spectroscopy and extensive NMR techniques, in conjunction with 2D-NMR experiments.
3) Evaluation of biological activities using selective pharmacological models in cell lines and/or selective *in vitro* assays.
4) Prediction of the biological properties by chemi-informatic tools and generation of database of potential lead molecules
5) Development of product/s as nutraceutical(s)

Review of Literature:

Cephalopods were considered as significant resources of bioactive metabolites with prominent bioactive potentials (Krishnan et al., 2019). Knowledge of bioactive compounds from these organisms would help to develop newer pharmacological agents. Secondary metabolites classified under different classes, such as spirocyclics, oxygenated heterocyclics, polysaccharides, macrocyclic lactones, steroids and polyketides with promising biological functions (Blunt et al., 2016).

Cephalopoda are used in a wide range of traditional medicines, but there are relatively few chemical investigations on isolation of biologically active metabolites. *Sepia* ink has been used for the treatment of depression in china (Cazalet, 2007) and it is listed in the homeopathic *Materia Medica*. Four cephalopod species were listed in a survey of South African medicines; the internal shells of the species *Spirula spirula* were the most expensive marine invertebrate on sale at the traditional medicine market in Durban (Herbert et al., 2003). Cuttlefish, *Sepia officinalis* have been reported to inhibit of acetic acid-induced writhing and increase in latency period of mice in the hotplate (Fahmy & Soliman, 2013). Cuttlefish, *Sepiella inermis* displayed ACE-1 inhibitory activity, anti-diabetic activities of inhibition of α-amylase and dipeptidyl peptidase 4 enzymes (Krishnan et al., 2019). *Amphioctopus marginatus* shows antioxidant activity, anti-hypertensive activity against ACE-1 and HMGCo-enzyme reductase inhibition. Cuttlefish consumption reportedly alleviates conditions like inflammation of ear and diseases of the digestive tract (Kim & Pallela, 2012). The medicinal

applications of these species remain unknown and they are yet to be subjected to any detailed natural product investigations.The most advanced and organized class of the phylum Mollusca, Cephalopods are distributed throughout the seas of the world. Over the past decades, Cephalopods are respected for various contributions in scientific research. However, very few studies have been conducted in isolating secondary metabolites from cephalopods.

Most of the works were carried out on their physiology, bioluminescence, mimicry and ink productions (Kornprobst, 2010). Adenochromines were reported from Octopus vulgaris (Prota et al.,1977). Arsenolipids were reported from a squid, Todarodes pacificus (Ninh et al., 2007). Cyclophosphamine extracted from squid ink was a well-known chemotherapeutic drug (Zhong et al., 2009). Cytotoxic tyrosinase was isolated from S. officinalis (Russo et al., 2003). Novel cardioactive peptides were purified from the Japanese octopus, Octopus minor (Iwakoshi et al., 2000). Astaxanthin and its ester derivatives were found to be the major carotenoids in octopus and cuttlefish species (Maoka et al.,1989). The Octopodidae cephalopod, A. neglectus was reported to contain four macrocyclic lactones with antihypertensive potencies, among which macrocyclic lactones with furo[1,4,8]trioxacyclohexadecine-12, 19-dione functionality, exhibiting potent protective properties against angiotensin-II induced cardiac hypertrophy at 25 µg/mL on the H9C2 cell lines in the ex-vivo analyses (Chakraborty et al., 2019a). Spineless cuttlefish, S. inermis was found to possess four chromenyl derivatives, named as methyl 7-ethyl-hexahydro-8a-methyl-2H-chromene-4-carboxylate and methyl 1-acetoxy-hexahydro-3-methyl-3-propyl-1H-isochromene-4-carboxylate with selective in vitro antioxidative/anti-inflammatory potentials (Krishnan et al., 2018). Three antioxidative oxygenated terpenoids were reported from U. duvaucelii with anti-inflammatory activities, and their structural attributions elucidated as C19 furano-norditerpenoid, irregular C15 sesquiterpenoid and C20 diterpenoid (Chakraborty et al., 2019b).

Chakraborty et al., 2019a reported four macrocyclic lactones analogues from *Amphioctopus neglectus* with inhibitory properties against Angiotensin converting enzyme (ACE), in which compound **2** exhibit potent inhibitory activity against Ag-II induced cardiac hypertrophy on H9C2 cells and hence could be utilised to develop potential lead against hypertension and related ailments. Bioactivity guided chromatographic purification of extract of *Amphioctopus marginatus* led to the identification of anicosahydropicene, sterols and octahydroazuleno-pyran-dione based derivatives (Chakraborty & Joy 2018). Arsenolipids, which are both water and lipid-soluble arsenic compounds, has been reported from liver phospholipids of Japanese flying squid *Todaro despacificus.* The major arsenolipids isolated were dimethyl arsinic acid (DMA)-containing glycerophospholipid (phosphatidyl dimethylarsinic acid) and DMA-containing sphingomyelin (Ninh et al., 2007). The pigments, adenochromines were discovered from *Octopus vulgaris* (Ito et al., 1976; Prota et al., 1977). Three oxygenated terpenoids with bioactivities against pro-inflammatory inducible enzymes were isolated from Indian squid, *Uroteuthis* (Photololigo) *duvaucelii* (Chakraborty et.al. 2019b). Chemical examination of spineless cuttlefish *Sepiella inermis* resulted in the isolation of two anti-inflammatory chromenyl derivatives (Krishnan et al., 2018). Two chromenyl derivatives of potent antihyperglycemic agents attenuate serine protease dipeptidyl peptidase IV were isolated from spineless marine cuttlefish *Sepiella inermis* (Krishnan et al., 2019).

Cephalopods are potential resources of $C_{20\text{-}22}$ long chain n-3 polyunsaturated fatty acids comprising eicosapentaenoic acid (EPA) and docosahexaenoicacid (DHA).These n-3 PUFAs are known to have variety of health benefits against, such as cardiovascular diseases (CVD's) including well-established hypotriglyceridemic and anti-inflammatory effects. Also, various studies indicate promising antihypertensive, anticancer, antioxidant, anti-depression, anti-aging, and anti-arthritis effects (Siriwardhana et al., 2012). PUFAs (n-3) are well known for their anti-inflammatory activity. The beneficial effect on anti-inflammatory property is attributed to its main mechanism of action of EPA as a competitive competitive inhibitor for pro inflammatory arachidonic acid (AA) on cyclooxygenase (COX) and Lipoxygenase (LOX) enzymes which eventually results in the generation of less inflammatory prostaglandin (PGE_3) instead of highly active pro inflammatory prostaglandin (PGE_2) due to is structural similarity with arachidonic acid (AA) (Calder 2009). The edible portion of Indian Ocean Squid *Uroteuthis duvaucelii* and Octopus *Amphioctopus neglectus* collected from Arabian sea found to exhibit greater content of n-3/n-6 polyunsaturated fatty acid ratio (10.64 ± 0.65 and 6.35 ± 0.31 respectively) and thus can be serve as a substitute to balance the greater admission of n-6 fatty acids in general utilization of vegetable oil (Chakraborty et al., 2016). Cephalopods are known for their lesser lipid content (0.92 to 1.58 g 100 g-1) as compared to other marine finfishes (Njinkoue et al., 2002). Earlier literature reported greater lipid content of *Amphioctopus marginatus* among various other cephalopod species collected from Cochin Fisheries Harbour (8°48'N; 78°9'E) along the south-west coast of India, which were lesser than those recorded in the edible portion of the marine fishes (Zlatanos et al., 2006). Calcium and phosphate is crucial for maintaining normal physiological condition and an imbalance of this regulation leads to

heart and chronic kidney disease (Blaine et al., 2015). A previous study of *Uroteuthis duvaucelii*, *Sepia inermis, Amphioctopus marginatus* and other common mollusc species, *Sepia inermis* displayed higher level of calcium and phosphorous (Ca+P) than other considered species and it should be noted that the digestive gland of cephalopods is capable of retention of high atomic weight elements, such as mercury (Hg), cadmium (Cd) and lead (Pb), likely as chelating agents with proteins (Venugopal and Gopakumar, 2017). Regarding trace metals, Zinc and copper occupies the dominant positions. Another study of *Amphioctopus neglectus, Cistopus indicus, Uroteuthis duvauceli, Sepia pharaonis and Sepiella inermis* was found to contain greater quantities of sulfur containing amino acids (23 mg/100 g) and lysine (36 mg/100 g) than other cephalopods, which indicated that the protein can effectively complement the limiting amino acids in our daily diets (Chakraborty et.al., 2016). Fatty acid based atherogenicity and thrombogenicity indices (<0.8 and <0.3, respectively) were found to be ideal in cephalopods ensuring that they can be consumed without any risk for health. Several studies have shown that cephalopod lipid profiles display high concentration of phospholipids, cholesterol and Polyunsaturated Fatty Acids (PUFA). Okuzumi and Fujii (2000) reported a content of cholesterol in several cephalopods such as 123 mg/100 g in cuttlefish, 180 mg/100 g in squid and139 mg/100 g in octopus. According to Nurjanah et al., 2012, cuttle fish contain essential nutrients for human good health with high in protein and mineral content, but low in lipid content. The body of cuttlefish contained high level of PUFA and various kinds of amino acid.

Methodology:

Sample collection and preparation of crude extracts

Samples of six economically important of cephalopods: *Cistopus indicus, Amphioctopus marginatus, Amphioctopus aegina*, *Sepia pharaonis, Sepia elliptica* and *Sepia aculeata* will be collected from marine ecosystems. The extracts/solvent fractions will be prepared from the selected cephalopods and will be evaluated for antioxidant, antihypertensive, anti-inflammatory, anti-diabetic and anti-hypercholesterolmic activities using various pharmacological models (Maneesh et al, 2016; Holmquist et al, 1979; Larsen et al, 1996; Baylac and Racine, 2003; Hamdan and Afifi 2004).

In vitro pharamocological profiling

The extracts/solvent fractions will be screened for antiradical activity by selective assays (Cotelle et al 1996) with suitable modifications. The anti-inflammtory activity will be screened by the method of Cheenpracha et al. (2010) using cyclooxygenase isoforms (COX-1, COX-2) and 5-lipoxygenase (5-LOX) attenuation potential. The anti-hypercholesterolemic, anti-dyslipidemic and antihypertensive activities will be screened using the methods of Rachmawati et al. (2016), Zhang et al. (2008) and Chaudhary et al. (2014), respectively.

Bioactivity-guided chromatographic purification of crude extracts

The crude extracts will be fractionated by column chromatography using various adsorbents. Chromatographic fractionation will be performed on vacuum liquid column/ flash column chromatography/preparative HPLC/gel permeation size chromatography over different adsorbents and buffers to yield various sub fractions. Crude polysaccharide extracted from the seaweed will be sequentially fractionated by anion exchange chromatography using resinous materials, and will be eluted with increasing salt gradient to yield the sub-fractions that will be subjected to total carbohydrate assay for positive chemical reaction of polysaccharide (Dubois et al 1956). Purified fractions will be freeze-dried before being subjected to HPLC analysis for monosaccharide composition analysis and purity by an evaporative light scattering detector (ELSD, SofTA 300S, Teledyne Technologies Inc., Lincoln NE). The homogeneity of the purified samples will be determined by chromatographic techniques. The eluted fractions will be evaluated for their antioxidant, antihypertensive, anti-inflammatory, anti-diabetic and anti-hypercholesterolmic activities.

Spectroscopic characterization of purified compounds and structure activity relationship analyses and molecular docking

The purified compounds will be characterized using extensive spectroscopic techniques like infrared (IR) spectroscopy, one-dimensional and two-dimensional nuclear magnetic resonance spectroscopy (NMR) techniques, ^{1}H and ^{13}C NMR, mass spectroscopy, ^{1}H-^{1}H correlation spectroscopy (^{1}H-^{1}H COSY), hetero nuclear single quantum coherence (HSQC), hetero nuclear multiple bond coherence (HMBC). Based on the carbohydrate content and monosaccharide compositional studies, the homogenous fractions will be characterized by spectroscopic experiments. Electrospray ionization (negative-ion mode)-coupled mass spectroscopic (ESI-MS) experiment of the hydrolyzed polysaccharide deduced the molecular weight. Structural composition and glycosidic linkage pattern of pure polysaccharide fractions will be identified with the aid of a modified method of methylation (Ciucanu and Kerek 1984) followed by a series of chemical derivatization procedures. The structural pattern of polysaccharide derivatives will be elucidated by using gas-chromatographic mass-spectroscopic (GC-MS) analysis. The structural features and molecular parameters of the potential lead molecules responsible for the targeted bioactivities will be identified by structure/activity relationship analyses. The molecular interaction of purified compounds in the active site of enzymes through molecular docking analysis will be studied using molecular docking analyses.

To develop nutraceutical leads from the promising bioactive compounds

Method/s and protocol/s to prepare nutraceutical product(s) with enriched bioactive fractions will be developed and standardized. Optimized protocols will be developed to scale-up the yield of bioactive leads and assessing their stability by accelerated shelf-life studies.

Expected outcome:

- Database of prospective bioactive small molecular weight leads and polysaccharides from cephalopods of marine origin with anti-hypertensive, anti-inflammatory, anti-dyslipidemic and anti-diabetic activities.
- Nutraceutical product(s) will be developed.

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